CB2/miR-124 signaling down-regulate the expression of purinergic $P2X_4$ and $P2X_7$_receptor in dorsal spinal cord of CCI rats

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Research

Keywords: CB2 receptor, P2X4 receptor, P2X7 receptor, dorsal spinal cord, neuropathic pain

Posted Date: January 28th, 2020

DOI: https://doi.org/10.21203/rs.2.22085/v1

Abstract

Background:

The importance of P2X purinoceptors, CB2 receptor and microRNA-124(miR-124) in spinal cord microglia to the development of neuropathic pain was demonstrated in numerous previous studies. The upregulation of $P2X_4$ and $P2X_7$ receptors in spinal dorsal horn microglia is involved in the development of pain behavior caused by peripheral nerve injury. However, it is not clear whether the expression of $P2X_4$ and $P2X_7$ receptors at dorsal spinal cord will be influenced by CB2 receptor or miR-124 in rats after chronic sciatic nerve injury.

Methods: Chronic constriction injury (CCI) of the sciatic nerve was performed in rats to induce neuropathic pain. Tests of the mechanical withdrawal threshold (MWT) were carried out to assess the response of the paw to mechanical stimulus. The expression of miR-124, $P2X_4$, $P2X_7$ and CB2 receptor were detected with RT-PCR. The protein expression of $P2X_4$, $P2X_7$ and CB2 receptor, RhoA, ROCK1, ROCK2, p-p38MAPK and p-NF-kappaBp65 was detected with Western blotting analysis.

Results:

Intrathecal administration of CB2 receptor agonist AM1241 significantly attenuated CCI-induced mechanical allodynia and significantly inhibited the increased expression of $P2X_4$ and $P2X_7$ receptors at the mRNA and protein levels, which imply that $P2X_4$ and $P2X_7$ receptors expression are down-regulated by AM1241 in CCI rats. Western blot analysis showed that AM1241 suppressed the elevated expression of RhoA, ROCK1, ROCK2, p-p38MAPK and NF-κBp65 in the dorsal spinal cord induced by CCI. After administration with Y-27632 (ROCK inhibitor), SB203580 (P38MAPK inhibitor) or PDTC (NF-κB inhibitor), the levels of $P2X_4$ and $P2X_7$ receptors expression in the dorsal spinal cord were lower than those in CCI rats, which imply that the ROCK/P38MAPK pathway and NF-κB activation may contribute to the increased expression of $P2X_4$ and $P2X_7$ receptor. On the other hand, in CCI rats, AM1241 treatment evoked the increased expression of CB2 receptor and miRNA-124, which can be inhibited by intrathecal injection of CB2 receptor antagonist AM630, which indicate that the increased expression of miRNA-124 may be medicated by CB2 receptor activation. In addition, the increased expression of $P2X_4$ and $P2X_7$ receptors in the dorsal spinal cord of CCI rats were inhibited by miRNA-124 agomir. Furthermore, intrathecal injection of miRNA-124 agomir could efficiently inhibit the ROCK/P38MAPK pathway and NF-κB activation in CCI rats. Moreover, AM1241 treatment significantly inhibited the expression of $P2X_4$ and $P2X_7$ receptors, and this suppression is enhanced by pretreatment with miRNA-124 agomir. On the contrast, the inhibitory effect of AM1241 on the expression of $P2X_4$ and $P2X_7$ receptor can be reversed by pretreatment with miRNA-124 antagomir.

Conclusions: In CCI rats, intrathecal injection of AM1241 could efficiently induce the increased expression of miRNA-124, while inhibiting the ROCK/P38MAPK pathway and NF-κB activation in dorsal spinal cord.

CB2 receptor/miRNA-124 signaling induced the decreased $P2X_4$ and $P2X_7$ receptors expression via inhibit the ROCK/P38MAPK pathway and NF-κB activation.

Background

It is well known that chronic neuropathic pain is often induced by peripheral nerve injury or dysfunction of the sensory nervous system in a large number of patients. Cannabinoids-mediate analgesia effect has been observed in various types of neuropathic pain in animals, including chronic constriction nerve injury, trigeminal neuralgia, chemotherapy- and streptozotocin-induced neuropathy [1]. More recent studies have suggested that cannabinoid CB1 and CB2 receptors, expressed in dorsal spinal cord, play an important role in spinal nociception [2-5]. Presynaptic inhibition may contribute to the analgesic effects of spinal CB1 activation [3]. CB2 receptor agonist-induced analgesia effect is associated with restriction in elevation of microglial density and elevation in phosphorylated P38mitogen-activated protein kinase (P38MAPK) in dorsal spinal cord microglia cells [4]. Administration of CB2 receptor agonist reduced the number of hypertrophic microglia while leaving microglial cell number unaffected. Furthermore, pro-inflammatory cytokines accumulation was obviously suppressed by CB2 receptor agonist in dorsal horn of in neuropathic pain models [5]. It looks like that dorsal spinal cord microglia is an important site for CB2 receptor-mediated analgesia.

A growing body of evidence shows that spinal microglia react and undergo a series of changes that is responsible for the establishment of neuropathic pain [6]. Spinal microglia cells express several P2 receptor subtypes including $P2X_4$, $P2X_7$, $P2Y_{12}$ and $P2Y_{13}$ receptors subtypes [7, 8]. The roles of these spinal cord P2 receptors in the transmission of pain information have been demonstrated in previous studies. Trang et al. found that the spinal $P2X_4$ receptor-evoked release and synthesis of brain-derived neurotrophic factor (BDNF) are necessary for maintaining pain hypersensitivity after nerve injury [7]. Spinal inhibition of $P2X_7$ receptor/ P38MAPK/IL-18 pathway reduced pin hypersensitivity in bone cancer- or chronic constriction injury-induced chronic pain [8, 9]. The elevated expression of $P2Y_{12}$ and $P2Y_{13}$ purinoceptors in spinal dorsal horn microglia is also involved in the development of pain behavior caused by peripheral nerve injury [10, 11]. In addition, we noticed that the interaction between cannabinoid and P2 purinoceptors in nervous system has been reported in recent studies. The analgesic effect of CB1 receptor activation is mediated by a negative modulation of the $P2X_3$ receptor in the primary afferent neurons in carrageenan-induced inflammatory pain in mice [12]. In cultured dorsal spinal neurons, the opening of P2X receptor channels is down-regulated by activation of cannabinoid CB1 receptor [13]. Our previous studies indicated that CB2 receptor activation produces a pronounced inhibition of CCI-induced thermal hyperalgesia and significantly inhibited the increased expression of $P2Y_{12}$ and $P2Y_{13}$ receptors, which open up the possibility that $P2Y_{12}$ and $P2Y_{13}$ receptor expression are down-regulated by CB2 receptor activation [11]. Based on these reports, we hypothesized that the expression of $P2X_4$ and $P2X_7$ receptor in dorsal spinal cord may be also influenced after CB2 receptor activation in neuropathic pain rats.

It is clear that microRNAs (miRNAs) can regulate expression of multiple target genes by promoting degradation of mRNA or by preventing translation of target genes. Recently, more and more studies suggested that, in dorsal root ganglia, spinal cord, nucleus accumbens, medial prefrontal cortex, periaqueductal gray and hippocampus, altered miRNA expressions are linked to the development of chronic pain [14-16]. The high levels of the miR-124 are present in resident microglia in dorsal spinal cord [17, 18]. The decreased expression of miR-124 in dorsal spinal cord and hyperalgesia were observed in neuropathic pain models [17-18]. More importantly, intrathecal miR-124 treatment repressed the development of neuroinflammation in neuropathic pain animals [17-19]. MiR-124 attenuated neuropathic pain by shifting spinal microglia M1/M2 polarization toward the M2 phenotype [17, 18]. On the other hand, peripheral nerve injury induced the appearance of CB2 expression in activated microglia cells in dorsal spinal cord [20]. Moreover, CB2 receptor seems to play an important role in the regulation of nerve injury-induced neuroinflammation within dorsal spinal cord [21]. These interesting experimental results promote us to further explore the role of miR-124 in CB2 receptor-mediated analgesia. At the same time, there is another question that whether the decreased miR-124 expression contributed the increased expression of $P2X_4$ and $P2X_7$ receptor in dorsal spinal cord in neuropathic pain rats.

Methods

Animals

All experiments were performed on male Sprague-Dawley rats, weighing 180-220 g. All rats were obtained from Zunyi Medical University Animal Laboratory Center (Guizhou, China) and were kept in cages lined with sawdust, bred in a 12-h light-dark cycle room with controlled temperature (25-27℃). All rats had access to water and food available ad libitum. The Committee for Ethical Use of Zunyi Medical University approved all the experiments as per relative international codes.

Intrathecal injection of drugs

Lumbosacral intrathecal catheters were constructed and implanted as detailed in our previous studies [11, 22, 23]. Under anesthesia with pentobarbital sodium (40 mg/kg, i.p.), rats were fixed and 2-cm longitudinal incision was made above vertebrae L_{4-6}. A PE-10 catheters (length: 15cm; inner/outerdiameter:0.28/0.61mm) were pushed through the intervertebral space until a sudden movement of tail or the hind limb were observed and then passed gently 2 cm upward to reach the level of the lumbar enlargement. The tip of the catheter was fixed on the neck area of the rats. Correct intrathecal placement was confirmed by injection of 2% lidocaine (10μl) through the catheter. The catheter was judged to be intrathecal if paralysis and dragging of bilateral hind limbs occurred within 30 s of this injection. In addition, rats with signs of motor weakness were excluded from the experiment. The rats were allowed to recover for 5 days before the CCI operation. Rats that exhibited motor deficits such as hind-limb paralysis, impaired righting reflexes, and hind-limb dragging were excluded.

AM1241 (CB2 receptor agonist, cat. no.ab120934, abcam), SB203580 (P38MAPK inhibitor, cat. no.ab120162, abcam) and PDTC (NF-κB inhibitor, cat. no.ab141406, abcam) were dissolved in dimethyl sulfoxide (DMSO) and then further diluted with 0.9% Sodium Chloride, respectively. Y-27632 (ROCK inhibitor, cat. no. Y0503, Sigma) was dissolved in 0.01M PBS. The presence of DMSO (<0.1%) did not affect the mechanical withdrawal threshold of the rat hind paw. miRNAs are small molecules with a short half-life and are easily degraded. MiR-124 agomir/ antagomir are a chemically modified miR-124 mimic/inhibitor that are more stable and has a higher cell membrane affinity than does the endogenous miRNA. MiR-124 agomir (Sense: 5'-UAAGGCACGCGGUGAAUGCC-3'; Antisense: 5'-CAUUCACCGCGUGCCUUA UU-3'), microRNA124 antagomir (Sequence: 5'-GGCAUUCACCGCGUGCCUUA-3'); agomir NC (Sense: 5'-UUCU CCGAACGUGUCACGUTT-3'; Antisense: 5'-ACGUGACACGU UCGGAGAATT-3') and antagomir NC (Sequence: 5'-CAGUACUUUUGUGUAGUACAA-3') were purchased from Shanghai Sangon Biotech Co., Ltd (Shanghai, China). These microRNA agents were dissolved in DNase/RNase-Free water and then diluted with normal saline. These agents were injected twice a day for consecutively 7 days after CCI operation.

The chronic constriction injury (CCI) model

Under anesthesia with pentobarbital sodium (40 mg/kg, i.p.), the left sciatic nerve was exposed, and a 15-mm length of sciatic nerve proximal to the sciatic trifurcation was carefully dissected from the underlying tissue. Four loose ligatures (using 4.0 braided silk) were applied around the sciatic nerve at 1 mm intervals. The left sciatic nerve was only exposed but not ligated in sham-operated groups. At the end of the surgery, both the muscle layer and the skin layer were closed. The wound was disinfected with iodophor solution.

Mechanical allodynia

The mechanical withdrawal threshold (MWT) was evaluated by measuring the hind paw withdrawal response to von Frey filaments tested using previously described methods [22, 23]. The MWT was recorded on the day before and after surgery (at postoperative day 1, 3, 5, and 7). An electronic von Frey plantar aesthesiometer (IITC, Wood Dale, IL, USA) was used in the present study. Before tests, rats were given 15 min to habituate to the test environment. A rigid tip was applied against the midplantar surface of the left hind paw. The MWT was automatically recorded by the device and the cutoff was set at 60 g. The rigid tip was presented perpendicularly to the plantar surface, and brisk withdrawal or paw flinching were considered as positive responses. the digital number presented on the monitor was recorded as MWT. Three successive stimuli were applied, and MWT was represented by the mean values.

RT-PCR

Rats were terminally anesthetized with pentobarbital sodium (40 mg/kg) at the 7th day after CCI or sham surgery. The lumbar region of the ipsilateral dorsal spinal cord was dissected and placed into separate RNase free 1.5 ml eppendorf tubes at -80^{o}C. Total RNA was isolated by using the TRIzol (MRC Co., Cincinnati, USA) method after 24 h of PDGF-BB and Rg1 action. After purification, the RNA was eluted

using RNasefree water, and its concentration and purity were estimated using Spectrophotometer (Thermo Fisher Scientific). All 260: 280 absorbance ratios were in the range of 1.9–2.1. During RNA isolation, samples were also treated with DNase (Qiagen) to remove any contaminating genomic DNA. Subsequently, cDNA was synthesized from RNA using the SuperScript II reverse transcriptase kit (Invitrogen) according to the guidelines of the manufacturer. The specific primers are listed in Table 1. The final volume for qPCR was 20 µl of which 8 µl were H_2O, 10 µl mastermix (Life Technologies), 1µl assay-mix (Life Technologies) and 1µl cDNA. Each qPCR was done in duplicate. Real-time PCR was performed on a Linegene Real-time PCR detection system (Bioer Technology, China). The reactions conditions were: (1) 95 ^{o}C 8 min 1 Cycle; (2) 95 ^{o}C 15 s and 60 ^{o}C 1 min, 40 Cycles. Data were analyzed using the $2^{-\triangle\triangle CT}$ method [24].

Table 1 Primers used for RT-PCR

gene	Forward	Reverse
CB2R (NM-001164142.3)	5′-TGACCGCTGTTGACCGATAC-3′	5′-CAGGAGGTAGTCGTTGGGGAT-3′
$P2X_4R$ (NM-031594.1)	5′-GCACCTGTCCAGAGATTCCTGATAAG-3′	5′-AACACATCTTCCAGTCGCAACTCC-3′
$P2X_7R$ (NM-019256.1)	5′-GCTCTTCAGTAAGATCGTGCTATCCAG-3′	5′-GCTTGCTGTTGATGGCCTCTCC-3′
β-actin (NM-031144.3)	5′-AGCCATGTACGTAGCCATCC-3′	5′-ACCCTCATAGATGGGCACAG-3′
MiR-124 (NR-031865.1)	5′-GCGTAAGGCACGCGGTG-3′	5′-AGTGCAGGGTCCGAGGTATT-3′
U6 (NM-001014013.1)	5′-CCTGCTTCGGCAGCACA-3′	5′-AACGCTTCACGAATTTGCGT-3′

Western Blot Analysis

Rats were terminally anesthetized with pentobarbital sodium (40 mg/kg). The lumbar region of the ipsilateral spinal dorsal horn was rapidly dissected and rinsed in cold phosphate buffered saline then homogenized in 1ml ice-cold chilled radioimmunoprecipitation (RIPA) lysis buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 µg/ml aprotinin, 100 µg/ml phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 µM batimastat (BB-94) and 1% protease inhibitor cocktail (Roche). Protein concentration was determined using a Bio-Rad Protein Assay kit (cat. no. 5000002; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Total proteins were diluted in 4× loading buffer and were incubated at 100°C for 5 min. Samples (80 µg) were then loaded onto a loading gel and separated on a Bis-Tris gel (12% gel for CB2R, RhoA, p38MAPK, NF-κBp65, $P2X_4R$, $P2X_7R$, BDNF, IL-18, TNF-α and β-actin; 6% gel for ROCK1 and ROCK2). Separated proteins were transferred onto a nitrocellulose membrane (CB2R, $P2X_4R$, $P2X_7R$, p-p38MAPK, p-NF-κBp65 and β-actin: 20V for 1h; RhoA, BDNF, IL-18 and TNF-α: 20V for 30min; ROCK-1 and ROCK-2: 300mA for 3h). Nonspecific sites were blocked for 2 h at room temperature in fat-free milk solution [10% in 0.1% Tween-Tris-buffered saline (TTBS)]. Membranes were then incubated overnight at 4°C with the following rabbit polyclonal antibodies: anti-CB2R (cat. no. ab3561; 1:200; Abcam; 1:1000), anti-RhoA (cat. no. 10749-1-AP; 1:1000;

Proteintech), anti-$P2X_4$ (cat. no. ab99514; Abcam; 1:1000), anti-$P2X_7$ (cat. no. ab109054; 1:500; Abcam), anti-BDNF (cat. no. 66292-1-Ig; 1:500; Proteintech), anti-IL-18 (cat. no. sc-7954; 1:200; Santa Cruz), anti-TNF-α (cat. no. ab6671; 1:1000; Abcam), anti-p-P38MAPK (cat. no. ab45381; 1:1000; Abcam), anti-p-NF-κBp65 (cat. no. sc-166748; 1:400; Santa Cruz), anti-ROCK1 (cat. no. 21850-1-AP; 1:500; Proteintech) and anti-ROCK2 (cat. no. 20248-1-AP; 1:500; Proteintech). In addition, mouse monoclonal beta-actin antibody (cat. no. 60008-1-Ig; 1:10000; Proteintech) was used as a loading control. The secondary antibodies (goat anti-rabbit IgG: cat. no. A0208; goat anti-mouse IgG: cat. no.A0216, Beyotime Institute of Biotechnology) were diluted to 1:1000 and incubated for 1.5 h at room temperature. Subsequently, the membranes were developed using the enhanced chemiluminescence (ECL) reagent Beyo ECL plus (Beyotime Institute of Biotechnology). Images of the blots were captured using a ChemiDoc XRS system (Bio-Rad Laboratories, Inc.). The image was scanned and band intensity was semi-quantified using Quantity One software v4.52 (Bio-Rad Laboratories, Inc.).

Statistical analysis

All data are presented as means ± standard deviation. Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by least significant difference (LSD) post hoc test when multiple comparisons (SPSS19.0, SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

Results

Effects of AM1241 on mechanical allodynia and the expression of the CB2, $P2X_4$ and $P2X_7$ receptors in dorsal spinal cord of CCI rats

To evaluate the effect of AM1241 on mechanical allodynia in neuropathic and sham-operated rats, AM1241 was delivered intrathecally twice a day for postoperative days 1-7. Previous experiments have indicated that AM1241 at 100pM showed apparent elevations of the mechanical and thermal pain threshold, with maximal effect at 1.5 h after AM1241 administration [11, 25]. Then, we observed the MWT values at 1.5 h after AM1241 (100pM) administration. As shown in Fig.1A, there was no significant difference in the MWT among these groups before the operation. Decreased MWT were exhibited in the CCI rats on day 1 after nerve injury compared to sham rats ($P < 0.05$), and the allodynia was sustained for 7 days. However, sham-operated rats showed no obvious changes at all time points. Compared to the vehicle-treated CCI group, AM1241 at 100pM produced good anti-hyperalgesic effect as early as 1 day after CCI ($P < 0.05$), and the effects lasted for 7 days ($P < 0.05$).

It is well known that the increased expression of $P2X_4$ and $P2X_7$ receptors in dorsal spinal cord microglia cells were contributed to the nerve injury-induced microglia activation and neuropathic pain [7, 8]. It has been previously demonstrated that, in dorsal spinal cord of CCI model rat, the activation of microglia (with the marker Iba-1 or CD11b) and $P2X_4$ and $P2X_7$ receptor (both specifically expressed in microglia) expression were sharp increased at 7 days after sciatic nerve injury [26-27]. Then, to analysis the role of $P2X_4$ and $P2X_7$ receptors in the CB2 receptor-mediated analgesia effect, both mRNA and protein levels of

these two P2X receptors in dorsal spinal cord at 7 days after nerve injury were measured. As shown in Fig.1B, $P2X_4$ and $P2X_7$ receptors mRNA in dorsal spinal cord were elevated after nerve injury comparing to sham group ($P < 0.05$) and significantly down-regulated after repeated administration with 100pM AM1241 ($P < 0.05$). Zhang et al. reported that increased expression of CB2 receptor in rat spinal cord microglia in spinal nerve lesion model [28]. In agreement with the suggestion, we found the rats with sciatic injury also presented increased expression of CB2 receptor mRNA ($P < 0.05$) and the expression level was further enhanced after AM1241 treatment, which imply AM1241-induced CB2 receptor and downstream signaling pathway activation might further drive the receptor expression. Subsequently, the WB data further confirmed these results (Fig.1C and D). Compared with the sham group, the CCI rats displayed significantly increased protein expression of CB2, $P2X_4$ and $P2X_7$ receptors ($P < 0.05$). Compared with vehicle-treated CCI group, AM1241 treatment significantly further enhanced the protein expression of CB2 receptor in neuropathic pain rats (Fig.1D, $P < 0.05$). However, the increased protein expression of $P2X_4$ and $P2X_7$ receptors in dorsal spinal cord of CCI rats was significantly suppressed after repeated administration with AM1241 (Fig.1D, $P < 0.05$). These preliminary experimental results from RT-PCR and WB suggest that decreased expression of $P2X_4$ and $P2X_7$ receptors may be considered as one key element in CB2 receptor-mediated analgesia.

Effects of AM1241 on the expression of the BDNF, IL-18 and TNF-α in the dorsal spinal cord of CCI rats

Some previous studies reported that $P2X_4$ receptor-mediated synthesis and release of BDNF and tumor necrosis factor-α (TNF-α) in dorsal spinal cord microglia is necessary for maintaining pain hypersensitivity in nerve injury- and bone cancer-induced chronic pain [7, 29, 30]. $P2X_7$ receptor-dependent interleukin -18 (IL-18) and TNF-α production in dorsal spinal cord microglia plays an important role in some different chronic pain models (CCI, bone cancer pain, morphine analgesia) [8, 9, 30, 31]. Then, to further evaluate the functions of these two P2X receptors in CCI and AM1241-treated CCI rats, the expression of BDNF, IL-18 and TNF-α were observed. As shown in Fig. 2, at 7 days after nerve injury, dorsal spinal cord expression of BDNF, IL-18 and TNF-α in CCI rats was significantly higher than that in sham group ($P < 0.05$). Compared with CCI and vehicle-treated CCI rats, intrathecal administration of AM1241 significantly reduced the increased expression of BDNF, IL-18 and TNF-α in neuropathic pain rats ($P < 0.05$). It seems that AM1241 attenuates neuropathic pain may be through inhibiting these two P2X receptors-induced the production of BDNF, IL-18 and TNF-α in a rat model of chronic constriction injury. CB2 receptor-induced the expression down-regulation and functional impairment of $P2X_4$ and $P2X_7$ receptor may be one important mechanism of AM1241-mediated analgesia effect.

Effects of AM1241 on the expression of the RhoA, ROCK1, ROCK2, p-P38MAPK and p-NF-κBp65 in the dorsal spinal cord of CCI rats

Tatsumi et al. suggested the essential role of ROCK in nerve injury-induced P38MAPK phosporylation and mechanical hypersensitivity in rats with spared nerve injury (SNI) [32]. In addition, intrathecal

injection of the p38MAPK antagonist SB203580 decreased NF-κB activation, resulting in pain relief in CCI and SNI rats [11, 33]. However, it is not clear whether the expression of RhoA, ROCK1, ROCK2 and the activation of p38MAPK and NF-κBp65 is influenced after CB2 receptor activation in the development of neuropathic pain. Then, the expression of RhoA, ROCK1, ROCK2, p-p38MAPK and p-NF-κBp65 were detected by WB. As shown in Fig. 3, we noticed that, compared with the sham rats, the expression of RhoA, ROCK1 and ROCK2 in the CCI rats was significantly increased at 7 days after nerve injury ($P < 0.05$). AM1241 (100pM) treatment significantly reduced the expression of RhoA, ROCK1 and ROCK2 in rat dorsal spinal cord (7 days: $P < 0.05$) compared with that in the vehicle-treated CCI rats. Similar expression pattern of p-p38MAPK and p-NF-kappaBp65 is also observed in the current experiments. The CCI group displayed significantly increased p-p38MAPK and p-NF-κBp65 on days 7 compared with sham groups ($P <0.05$). After administration with AM1241 for 7 consecutive days, the expression levels of p-p38MAPK and p-NF-κBp65 in the spinal cord were lower than those in the CCI group ($P <0.05$). It looks like that AM1241-mediated analgesia effect occurred by inhibiting RhoA/ROCK expression and by decreaseing the phosphorylation of p38MAPK and p65 subunit of NF-κB. In addition, the decreased expression of RhoA, ROCK1 and ROCK2 might contribute to the decreased expression of p-p38MAPK and p-NF-κBp65 in AM1241-treated CCI rats.

Effects of Y-27632, SB203580, PDTC on the expression of $P2X_4$ and $P2X_7$ receptors in the dorsal spinal cord of CCI rats

In the present study, we found that CCI operation causes an increase expression in RhoA, ROCK1, ROCK2, p-P38MAPK and p-NF-κBp65 that also paralleled with the increased expression of $P2X_4$ and $P2X_7$ receptors in the dorsal spinal cord of CCI rats. However, it is not clear the role of ROCK, P38MAPK, NF-κB signaling pathway in $P2X_4$ and $P2X_7$ receptor expression in rats after nerve ligation. Thus, the ROCK inhibitor Y-27632 (10^{-5}mol/l), p38MAPK inhibitor SB203580 (50μmol/l) and NF-kappaB inhibitor PDTC (5μg/10μl) were applied in CCI rats. The MWT values and the expression of $P2X_4$ and $P2X_7$ receptors were detected after repeated intrathecal injection of these drugs. The CCI group displayed significantly decreased mechanical withdrawal threshold on days 1, 3, 5, and 7 compared with the sham rats (Fig.4A: $P < 0.05$), which were markedly increased by Y-27632, SB203580 or PDTC (Fig.4A: $P < 0.05$), respectively. In Fig.4B, compared with vehicle-treated CCI group, Y-27632, SB203580 or PDTC treatment significantly suppressed the increased expression of $P2X_4$ and $P2X_7$ receptors mRNA on day 7 after CCI surgery (Fig.4B: $P < 0.05$). At the same time, in Fig.4C and D, compared with vehicle-treated CCI group, Y-27632, SB203580 or PDTC treatment significantly suppressed the increased protein expression of $P2X_4$ and $P2X_7$ receptors after nerve injury ($P<0.05$). It appears that these results from RT-PCR and WB indicated the increased expression of $P2X_4$ and $P2X_7$ receptors may be regulated via ROCK/p38MAPK and NF-κB activation. But we are very curious what's causing the increased expression of RhoA, ROCK1, ROCK2, p-P38MAPK and p-NF-κBp65 expression in CCI rats.

Effects of Y-27632, SB203580, PDTC on the expression of BDNF, IL-18 and TNF-α in the dorsal spinal cord of CCI rats

It is well known that, in the neuropathic pain rats, the increased expression of BDNF, IL-18 and TNF-α in dorsal spinal cord microglia is related with $P2X_4$ and $P2X_7$ receptor activation [29-31]. To further explore whether the functions of these two P2X receptors is associated with ROCK/P38MAPK/NF-κB pathway in the development of neuropathic pain, Y-27632 (10^{-5}mol/l), SB203580 (50μM) or PDTC (5μg/10μl) were used and the expression of BDNF, IL-18 and TNF-α were observed. As shown in Fig. 5, compared with the sham group, the expression of BDNF, IL-18 and TNF-α in the CCI rats was significantly increased at 7 days after nerve injury ($P < 0.05$). Y-27632, SB203580 or PDTC treatment significantly reduced the protein expression of BDNF, IL-18 and TNF-α in the rat dorsal spinal cord ($P < 0.05$). These results indicate that Y-27632, SB203580 or PDTC could efficiently decreased the expression and function of $P2X_4$ and $P2X_7$ receptor, which results in the decreased production of BDNF, IL-18 and TNF-α in dorsal spinal cord of CCI rats.

Effects of CB2 receptor agonist/antagonist on the expression of miR-124-3p, $P2X_4$ and $P2X_7$ receptors mRNA in the dorsal spinal cord of CCI rats

More recent studies convince that miR-124 is one of the most abundant microRNAs in the brain and spinal cord that regulates microglial function [17, 18, 34, 35]. The decreased expression of miR-124 was reported in cancer pain, carrageenan-induced and peripheral nerve injury-induced chronic hyperalgesia [17, 18, 36, 37]. Intrathecal miR-124 application reversed the persistent hyperalgesia in these pain models. However, it is not clear whether miR-124 expression in dorsal spinal cord is associated with CB2 receptor-mediated analgesia effect. Then, AM1241 (CB2 receptor agonist) and AM630 (CB2 receptor antagonist) were used, respectively. As shown in Fig.6A, treatment with AM1241 reduced this CCI-induced mechanical allodynia ($P<0.05$, compare with CCI rats) and the effect was reversed by the AM630 ($P<0.05$, compare with AM1241-treated CCI rats). Meanwhile, these CCI rats displayed the decreased expression of miR-124 and increased $P2X_4$ and $P2X_7$ receptors mRNA in dorsal spinal cord ($P<0.05$, compare with sham group). Repeated AM1241 treatment for a period of 7 days resulted in the upregulation of miR-124-3p expression and the downregulation of $P2X_4$ and $P2X_7$ receptors mRNA in dorsal spinal cord ($P<0.05$, compare with CCI rats), which imply that CB2 receptor activation may probably drives the expression of miR-124. Besides, repeated AM630 pretreatment obviously reversed the AM1241-induced the increased expression of miR-124-3p and the decreased expression of $P2X_4$ and $P2X_7$ receptors mRNA ($P<0.05$, compare with AM1241-treated CCI rats), which further confirm that the elevated expression of miR-124 is at least in part due to CB2 receptor activation. Based on these results, it is reasonable to speculate that the spinal CB2 receptor activation may be an important mechanism of the increased expression of miR-124 and decreased expression of $P2X_4$ and $P2X_7$ receptors in AM1241-treated neuropathic pain rats. Of course, the preliminary experimental result also indicates that the increased expression of miR-124-3p is related to the CB2 receptor-mediated analgesia effect. And most importantly, these interesting

experimental results promote us to further explore the role of miR-124-3p in CCI-induced the increased expression of $P2X_4$ and $P2X_7$ receptors in the pathogenesis of neuropathic pain.

Effects of miRNA-124-3p agomir/antagomir on the expression of the $P2X_4$ and $P2X_7$ receptors, RhoA, ROCK1, ROCK2, p-P38MAPK and p-NF-κBp65 in the dorsal spinal cord of CCI rats

Willemen et al. suggest that intrathecal administration of miR-124 completely prevented the transition to persistent pain by normalizing spinal microglia activity (with the M1/M2 markers) in the spared nerve injury model [17]. A recent study from Zhang et al. found that inflammatory cytokine (IL-1β, IL-6, and TNF-α) protein expression in rats after CCI greatly increased and miR-124-3p mimics depressed inflammation cytokine levels [37]. Even more, similar phenomenon can be seen in lipopolysaccharide-incubated spinal microglial cells [37]. Then, to evaluate whether miR-124-3p -induced antinociception is associated with the expression of the $P2X_4$ and $P2X_7$ receptors, miR-124-3p agomir or antagomir were applied through the intrathecal tube. The MWT values and the expression of miRNA-124-3p, $P2X_4$ and $P2X_7$ receptors were observed. We are pleased to find that, compare with vehicle-treated CCI rats, miR-124-3p agomir treatment produced a significant relief of mechanical hypersensitivity. As shown in Fig.7, after miR-124-3p agomir treatment, the MWT measured on days 3, 5 and 7 was significantly higher than these vehicle-treated CCI rats ($P<0.05$, compare with CCI rats). On the other hand, CCI-induced the increased expression of $P2X_4$ and $P2X_7$ receptors at the mRNA and protein levels was significantly attenuated in rats treated with miR-124-3p agomir ($P<0.05$, compare with CCI rats). On the other hand, miR-124 antagomir at 100nM application slightly enhanced the increased expression of $P2X_4$ and $P2X_7$ receptors at the mRNA levels in CCI rats, but no significantly differences between CCI and miR-124 antagomir-treated CCI group. These experimental results from RT-PCR and WB revealed that, in these neuropathic pain rats, the increased expression of $P2X_4$ and $P2X_7$ receptors in dorsal spinal cord was probably because of a decreased level of miR-124.

Based on our experimental data, it was found that both CB2/miR-124 pathway and CB2/ROCK/P38MAPK/NF-KB pathway in dorsal spinal cord may involved in AM1241-induced the decreased expression of $P2X_4$ and $P2X_7$ receptors in CCI rats. But it is not clear the interaction between miR-124 and ROCK/P38MAPK and NF-KB activation in the transmission of pain information at the spinal cord level. We noticed that miR-124 could directly target RhoA mRNA and repress its expression in the process of neurogenic transdifferentiation of adipose derived mesenchymal stromal cells [38]. Downregulation of ROCK1 by miR-124 was also observed in U87MG human glioma cells, human colorectal cancer clls and SK-ES-1 cells [46-48]. Moreover, miR-124 modulates hepatocellular carcinoma cell aggressiveness by repressing ROCK2 expression [39]. MiR-124 mediates morphine inhibition of the innate immunity by directly targeting a subunit of NF-κB p65 and inhibits its expression [40]. Through the analysis of these data generated in previous reports, we speculate that miR-124 may down-regulate the activation of ROCK/P38MAPK and NF-KB, which results in the down regulation of $P2X_4$ and $P2X_7$ receptor expression. For this reason, the expression of RhoA, ROCK1, ROCK2, p-P38MAPK and p-NF-κBp65 were observed in the dorsal spinal cord following the intrathecal injection of miR-124-3p agomir

(100nM) or antagomir (100nM) in CCI rats. As shown in Fig. 7C and D, CCI-induced the increased expression of RhoA, ROCK1, ROCK2, p-p38MAPKand p-NF-κBp65 were significantly attenuated in rats treated with miR-124-3p agomir. On the other hand, miR-124-3p antagomir can not impair the expression of RhoA, ROCK1, ROCK2, p-p38MAPK in CCI rats. However, after miR-124-3p antagomir administration, the expression of p-NF-κBp65 has been enhanced in the CCI rats. It seems that miR-124 attenuates neuropathic pain and the increased expression of $P2X_4$ and $P2X_7$ receptors through inhibiting RhoA/ROCK/P38MAPK and NF-κB activation in a rat model of chronic constriction injury.

Effects of miRNA-124-3p agomir/antagomir on the expression of $P2X_4$, $P2X_7$ receptor, RhoA, ROCK, p-P38MAPK, P-NF-Kbp65 in the dorsal spinal cord of AM1241-treated CCI rats

In the above experiment, CB2 receptor activation can stimulate the increased expression of miR-124-3p and the following down regulation of ROCK/P38MAPK and NF-KB activation is required for the decreased expression of $P2X_4$ and $P2X_7$ receptors. To further confirm whether miRNA-124-3p is an important downstream target of CB2 receptor, the following experiments were conducted to test the idea. Firstly, we observed the effect of intrathecal injection of miR-124 agomir (100 nM, 30 min) or antagomir (100 nM, 30 min) on mechanical allodynia and the expression of the $P2X_4$ and $P2X_7$ receptors both at the mRNA and protein level in dorsal spinal cord of AM1241-treated CCI rats. As a result, we found that, in Fig. 8A, AM1241-treated CCI rats displayed significantly increased MWT on days 1, 3, 5 and 7 compared with that in CCI group, which was markedly enhanced by pretreatment with miR-124-3p agomir ($P < 0.05$). AM1241-induced the increased MWT was significantly reversed by pretreatment with miR-124-3p antagomir ($P < 0.05$). It looks like that AM1241-mediate analgesic effect may be through miR-124. Similarly, RT-PCR and WB analysis showed that miR-124-3p agomir pretreatment obvious enhanced AM1241-induced the decreased expression of $P2X_4$ and $P2X_7$ receptors at the mRNA and protein levels in dorsal spinal cord after nerve injury ($P < 0.05$). On the contrast, AM1241-induced the decreased $P2X_4$ and $P2X_7$ receptor expressions were significantly reversed after miRNA-124-3p antagomir pretreatment ($P < 0.05$). It appears that our results further suggest that the expression level of miR-124-3p is closely related to the AM1241-induced analgesic effect. Secondly, similar protein expression pattern of RhoA, ROCK, p-P38MAPK, p-NF-κBp65 were shown in Fig.8E, F, G, and H. Compare with AM1241-treated CCI rats, miR-124-3p agomir pretreatment obvious enhanced AM1241-induced the decreased expression of RhoA, ROCK, p-P38MAPK, p-NF-κBp65 in dorsal spinal cord after nerve injury ($P < 0.05$). On the contrast, AM1241-induced the decreased expression of ROCK, p-P38MAPK, p-NF-κBp65 were significantly impaired after miR-124-3p antagomir pretreatment ($P < 0.05$). These observations indicate that the CB2 receptor activation can stimulate the increased expression of miR-124 and the decreased expression of $P2X_4$ and $P2X_7$ receptors. Of course, the expression of $P2X_4$ and $P2X_7$ receptors may be regulated by miR-124/ROCK/P38MAPK and NF-κB signaling pathway. At last, the expression of BDNF, IL-18 and TNF-α were also observed. We found that, compare with AM1241-treated CCI rats, AM1241-induced the decreased expression of BDNF, IL-18 and TNF-α was obvious enhanced after pretreatment with miR-124 agomir ($P < 0.05$). The action of AM1241 on the expression of BDNF, IL-18 and TNF-α was obviously impaired after pretreatment with miR-124 antagomir (compare with AM1241-treated CCI rats, $P < 0.05$).

Discussion

Over recent decades, mounting studies have provided evidence that dorsal spinal cord microglia cells can be considered as an important responder to noxious stimulation. In the pathophysiological condition after peripheral nerve injury, dorsal spinal cord microglia cells are activated and that this activation contributes to the development of neuropathic pain behaviors. In general, activated microglia cells display the changes of miRNA (a class of non-coding regulatory RNAs) or neurotransmitter receptor protein that regulate cytokines release in the dorsal spinal cord in some different pain models [6-9, 17-21]. Cannabinoid CB2 receptor and purinergic $P2X_4$ and $P2X_7$ receptor expression are highly inducible on the reactive microglia in rat dorsal spinal cord following peripheral nerve injury. MiR-124-3p was significantly downregulated in rats in nerve injury- or bone cancer-induced neuropathic pain. But very little research has been done about the interaction between CB2, $P2X_4$, $P2X_7$ and miR-124-3p in the chronic pain transduction. Our present experiments demonstrated that repeated intrathecal injection of AM1241 can suppress mechanical pain response, ROCK/P38MAPK/NF-κB signaling pathway and $P2X_4$ and $P2X_7$ receptor expression induced by CCI operation. The expression of miR-124-3p can be promoted in AM1241-treated CCI rats. Subsequently, we found that repeated intrathecal injection of miR-124-3p agomir can suppress the expression of $P2X_4$, $P2X_7$ through down-regulation of the ROCK/P38MAPK and NF-κB signaling pathway. In a summary, it looks like that CB2 receptor activation can stimulate the increased expression of miR-124 and the following down regulation of ROCK/P38MAPK and NF-κB activation is required for the decreased expression of $P2X_4$ and $P2X_7$ receptors.

Some previous studies demonstrate that targeting CB2 receptors with selective agonists may exert an anti-inflammatory effect in various inflammatory diseases. For example, in human endothelial cell and monocytes, TNF-α -induced NF-κB and RhoA activation were attenuated by CB2 agonist [41, 42]. In human astrocytes, CB2 agonist suppressed IL-1β-triggered the production of CX3CL1 and the effect is mediated through inhibiting p38MAPK signaling pathways [43]. The anti-inflammatory effect of CB2 agonist on the mice with experimental colitis is at least partially by inhibiting p38MAPK [44]. In microglia, upregulation of CB2 receptor has been associated with the restoration of tissue homeostasis in pathological neuroinflammatory condition [45]. Xiang et al. also reported that the modulation of microglia polarization may involve multiple mechanisms, mainly, the inhibition of NF-κB and MAPK activation [47]. In the present study, the increased expression of RhoA, ROCK1, ROCK2, p-P38MAPK and p-NF-κBp65 in CCI rats were significantly suppressed after AM1241 pretreatment. In addition, Tatsumi et al. suggest that ROCK activation is necessary for P38 MAPK phosphorylation in the development of chronic pain [31]. Furthermore, it was also reported by Wilkerson et al. that AM1241 produced profound anti-allodynia with a corresponding decrease in phosphorylation of p38MAPK immunoreactive cells and their protein levels in nonneuropathic pain rats [34]. Intrathecal injection of the p38MAPK antagonist SB203580 decreased NF-κB, resulting in pain relief in neuropathic pain rats [11, 36]. It appears that the inhibition of P38MAPK and NF-κB activation is important mechanism of AM1241-mediated analgesia effect. Our results are partly in agreement with those of Guo et al., who concluded that CB2 receptor activation suppressed hypoxia-induced neuroinflammatory response through inhibition of NF-κB activation in microglia [48].

Subsequently, we found that the inhibition of ROCK/P38MAPK and NF-κB and activation can obviously suppressed the increased expression of the increased expression of $P2X_4$ and $P2X_7$ receptor, which imply that increased expression of ROCK, phosphorylation of P38MAPK and activation of NF-κB may play an important role in CCI-induced the increased expression of $P2X_4$ and $P2X_7$ receptors. CB2 receptor mobilization can suppressed thses two P2X receptors expression by downregulating the ROCK/P38MAPK and NF-κB activation. Wu et al. suggested that paclitaxel-induced $P2X_4$ receptor expression and BDNF release in dorsal spinal cord microglia cells can be inhibited by a highly selective CB2 agonist MDA7 [49]. However, Masuda et al. reported that IRF8-IRF5 transcriptional axis may contribute to shifting spinal microglia toward a $P2X_4$ receptor-expressing reactive state after nerve injury [50]. On the other hand, in human chondrocytes, NF-κB was involved in the effects of IRF-5 on MMP-3 expression [51]. Further more, IKKβ activation can lead to the nuclear translocation of IRF5 and induction of inflammatory cytokines in human monocytes [52]. IRF5 can directly bind to DNA in the 5' upstream region of the TNF, its recruitment to the 3' downstream region of the TNF gene depends on the protein-protein interactions with NF-κB RelA [53]. Similarly, IRF5 binds to a noncanonical composite PU.1:ISRE motif, and its recruitment is aided by RelA in macrophages [54]. It seems that the next important question as to whether the interaction of NF-κB and IRF5 can enhance the expression of these two P2X receptors (in situ) awaits resolution.

Non-coding RNAs, such as microRNAs (miRNAs) that function as translational repressors are an important regulatory element in neurological disorders including Parkinson's disease, Alzheimer's disease, cerebral ischemia injury and chronic pain. Recently, the role of MiR-124 in neurological disorder has attracted remarkable attention. Furthermore, RHOA, ROCK1, ROCK2, p38MAPK and p-NF-κB P65 have been identified as the targets of miR-124 in previous studies [38-40, 46-48, 55]. Moreover, miR-124 could suppress p-p38 expression and could attenuate the activation of microglia in LPS-treated BV2 cells and the substantia nigra par compacta of MPTP-treated mice [55]. In the present study, repeated intrathecal injection of miR-124 can suppress mechanical pain response, ROCK/P38MAPK signaling and NF-κB activation induced by CCI operation. It is very clear that ROCK/P38MAPK signaling and NF-κB activation plays can promote the dorsal spinal cord microglia activation, which lead to the development of nueropathic pain [32, 33]. Addtionaly, the $P2X_4$ and $P2X_7$ receptor expression is mediated by ROCK/P38MAPK signaling and NF-κB activation in dorsal spinal cord. It appears that decreased expression RHOA, ROCK1, ROCK2, p-p38MAPK and p-NF-κBp65 may contribute to miR-124-medicated analgesia effect and the decreased expression of these two P2X receptor expressions.

In the present study, both CB2 receptor activation- and miR-124-medicated analgesia effect are mediated through down-regulating ROCK/P38MAPK signaling and NF-κB activation in CCI operation-induced neuropathic pain. We further explored whether the expression of miR-124 can be influenced by CB2 receptor activation. As a result, we found repeated AM1241 treatment resulted in the upregulation of miR-124 expression and the downregulation of $P2X_4$ and $P2X_7$ receptors mRNA in dorsal spinal cord, which imply that CB2 receptor activation may probably drives the expression of miR-124. Besides, AM630 pretreatment obviously reversed the AM1241-induced the increased expression of miR-124, which further confirmed the idea that the miR-124 expression may be medicated by CB2 receptor activation. Some

previous researches suggest that the expression level of miR-124 is regulated by different protein kinases or transcription factors. For example, the activated SMAD4 signal promotes the expression of miR-124-3p by SMAD4 binding to the promoter region of miR-124-3p in porcine preadipocytes. [59]. The phosphorylated level of AMP-activated protein kinase alpha (AMPKα) and the transcriptional activity of activator protein 2 alpha (AP-2α) can lead to the increased miR-124 expression in vascular smooth muscle cells [62]. Over expression of Gli2 (glioma-associated oncogene 2) suppresses miR-124 expression by increasing the direct binding of Gli2 to the upstream region of the transcriptional start site for miR-124 in glioma cells [63]. Among these protein kinase and transcription factors, the role of AMPK in spinal cord glial activation has been received attention in recent years. In CCI rats, phosphorylated-AMPK was expressed at lower levels in the dorsal spinal cord [64]. Moreover, activation of AMPK can effectively inhibit the activation of spinal microglia and astrocytes, which result in pain relief [64]. Lidocaine suppressed morphine-induced activation of spinal cord microglia and downregulated inflammatory cytokines production by activating AMPK [65]. On the other hand, it is well known that CB2/AMPKsignaling pathway was reported in some previous studies. For example, Wang et al. suggested that CB2R/AMPK/GSK3β pathway can be a promising new drug target for AD [66]. In cerebral ischemic injury model and in rat cortical neurons/glia mixed cultures, CB2 receptor agonist reduced mitochondrial dysfunction and intracellular oxidative stress and the effect is mediated through modulation of AMPK/CREB signaling [67]. Based on the above analysis, we speculate that CCI-induced the decreased expression of AMPK might contribute to the decreased expression of miR-124 in dorsal spinal cord. Besides, CB2 receptor/AMPK signaling pathway may play a role in AM1241-induced the elevated miR-124 expression. Of course, this assumption needs to be confirmed by further studies. Moreover, some previous reports indicated that DNA methylation can regulate microRNAs gene expression [68]. The DNA methylation profile of the miR-124 promoter region should be explored in the future.

In the central nerve system, spinal cord microglia cells are considered as the primary responders to noxious stimulation. In the spinal cord, CB2 receptor activation may inhibit the microglia ROCK/p38MAPK and NF-κB activation to prevent CCI-induced chronic pain. CB2 receptor stimulation decreased $P2X_4$ and $P2X_7$ receptor expression via miRNA-124-mediated the down regulation of ROCK/p38MAPK and NF-κB activation. In a word, CB2 receptor/miRNA-124 pathway may involved in AM1241-induced the down regulation of ROCK/p38MAPK and NF-κB activation, the decreased expression of $P2X_4$ and $P2X_7$ receptor expression, which result in the pain relief. It seems that decreased expression of $P2X_4$ and $P2X_7$ receptors may be considered one of the mechanisms involved in CB2 receptor-mediated analgesia.

Conclusions

In the present study, we found that CB2 receptor activation significantly inhibited peripheral nerve injury-induced thermal hyperalgesia and the increased expression of $P2X_4$ and $P2X_7$ receptors in the rat dorsal spinal cord. The suppression of ROCK/p38MAPK/NF-kappaB signaling may play a role in CB2 receptor-mediated analgesia. CB2 receptor stimulation induced the increased expression of miRNA-124, which

lead to the downregulation of ROCK/p38MAPK and NF-kappaB activation. Subsequently, decreased expression of $P2X_4$ and $P2X_7$ receptors were decreased. In summary, CB2 receptor/miRNA-124 pathway may involved in AM1241-induced the down regulation of ROCK/p38MAPK and NF-κB activation and the decreased expression of $P2X_4$ and $P2X_7$ receptor expression, which result in the pain relief. CB2 receptor/ miRNA-124 pathway induced the decreased expression of P2X4 and P2X7 receptors may be important for pathophysiological events occurring within the spinal cord, for where it is implicated in the transduction of the “pain” message.

Abbreviations

AD: Alzheimer's disease; AM1241: (2-Iodo-5-nitrophenyl)[1-[(1-methyl-2-piperidinyl)methyl]-1H-indol-3-yl] methanone; AMP: adenosine monophosphate; AMPK: AMP-activated protein kinase; AMPKα: AMP-activated protein kinase alpha; one-way analysis of variance (ANOVA); activator protein 2 alpha (AP-2α); AM630: [6-Iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl) methanone; BV2: the immortalized murine microglial cell line; BDNF: brain-derived neurotrophic factor; CCI: chronic constriction injury; CB1R: cannabinoid receptor 1; CB2R: cannabinoid receptor type 2; CREB: cAMP-response element binding protein; DMSO: dimethyl sulfoxide; Gli2: glioma-associated oncogene 2; GSK3β: glycogen synthase kinase 3 beta; Iba1:Ionized calcium–binding adaptor molecule-1; IKKβ: Inhibitor of nuclear factor kappa-B kinase subunit beta; IL-18: Interleukin-18; i.p.: Intraperitoneal injection; IRF5: interferon regulatory factor 5; IRF8: interferon regulatory factor 8; LPS: Lipopolysaccharides; MDA7: Melanoma differentiation-associated gene 7; MMP-3: matrix metallopeptidase 3; MPTP: Pyridine, 1,2,3,6-tetrahydro-1-methyl-4-phenyl-, hydrochloride (1:1); MWT: mechanical withdrawal threshold; MAPK: Mitogen-activated protein kinase; NF-κB: Nuclear factor-κB; PBS: Phosphate-buffered saline; PDTC: Pyrrolidine dithiocarbamate; P38MAPK: P38 mitogen activated protein kinases; RhoA:Ras gene family members A; RIPA: radioimmunoprecipitation; ROCK-1:Rho associated coiled-coil forming protein kinase 1; ROCK-2: Rho associated coiled-coil forming protein kinase 2; RT-PCR: Real time-Polymerase Chain Reaction; Real-Time RT-PCR: Real-time reverse transcription polymerase chain reaction; SNI: spared nerve injury; SMAD4: mothers against decapentaplegic homolog 4; SB203580: 4-[4-(4-Fluorophenyl)-2-[4-(methylsulfinyl)phenyl]-1H-imidazol-5-yl]pyridine; TNF-α:Tumor necrosis factor α; TTBS: Tween-Tris-buffered saline; TWL: thermal withdrawal latency; WB: Western blot; Y-27632: (R)-(+)-trans-4-(1-Aminoethyl)-N-(4-pyridyl)cyclohexanecarboxamide dihydrochloride;

Declarations

Acknowledgments

We are very grateful to the other staff of the Department of Physiology in Zunyi Medical College.

Funding

This work is supported by the National Natural Science Foundation of China (No. 31640040 and31860291), Major Research Projects for Innovative Group in Guizhou Province of China [No: 2018(025)] and Science and Technology Foundation of Guizhou Province of China [No: 2010(2264)].

Availability of data materials

There is no data, software, databases, and application/tool available apart from the reported in the present study. All data is provided in manuscript.

Authors' contributions

Rui Xu and Fan Yang drafted the manuscript. Rui Xu, Lijuan Li and Xiaohong Liu performed the CCI and behavioral assessment of pain. Xiaolu Lei and Tao Xu performed the Intrathecal catheterization and drug delivery. Zhi Xiao and Zucai Xu performed the RT-PCR and Western blot and analysis. Liu XH and Zeng JW conceived of the study, participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval

All animal procedures performed in this study were carried out in accordance with the guidelines of the International Association for the Study of Pain and were approved by the Zunyi Medical University Committee on Ethics in the Care and Use of Laboratory Animals.

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Figures

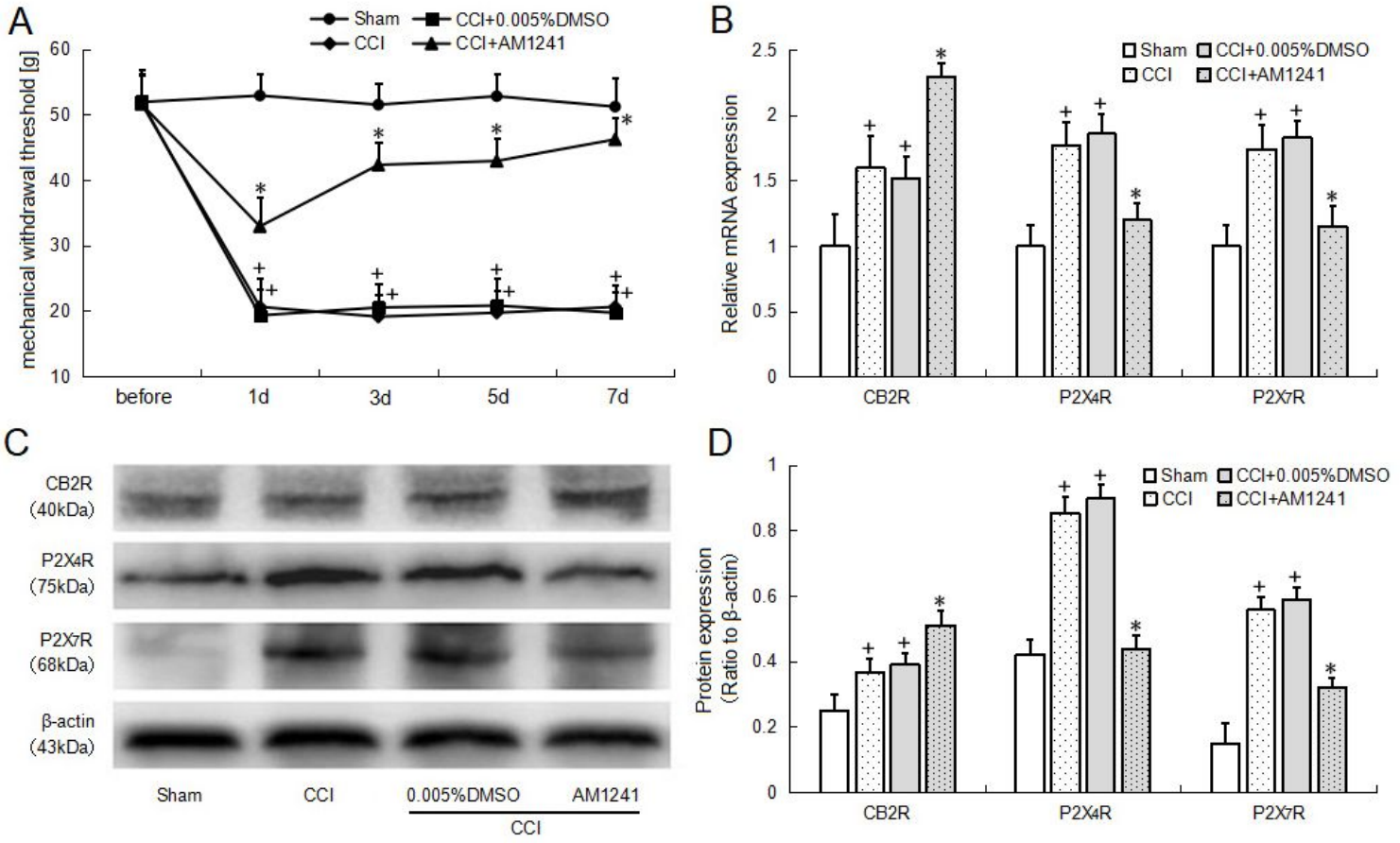

Figure 1

Fig.1 The effects of AM1241 on the mechanical allodynia and the expression of CB2, P2X4 and P2X7 receptors. All values represent mean ± SD. A: MWT in the ipsilateral hind paw was determined in different groups (n = 8). Decreased MWT was exhibited in CCI rats after surgery compared to sham rats (+P < 0.05). The values of MWT was higher in AM1241 (100pM)-treated CCI rats than that in the CCI group (*P < 0.05). B: RT-PCR results show the expression of CB2, P2X4 and P2X7 receptor mRNA expression in dorsal spinal cord (n = 8). The expression levels of CB2, P2X4 and P2X7 receptor mRNA in the CCI group was higher than that in the sham group (+P < 0.05). The expression levels of P2X4 and P2X7 receptor mRNA were significantly lower in AM1241 (100pM)-treated CCI rats than that in the CCI group (*P < 0.05). After AM1241 treatment, the expression levels of CB2 receptor mRNA was more elevated than that in the CCI group (*P < 0.05). C: Western blotting image of CB2, P2X4 and P2X7 receptors expression. D: Western blotting quantitative analysis of the CB2, P2X4 and P2X7 receptors expression in dorsal spinal cord (n=5). The protein expression levels of CB2, P2X4 and P2X7 receptors in the CCI group were higher than that in the sham group (+P < 0.05). The protein expression levels of P2X4 and P2X7 receptors were significantly lower in AM1241 (100pM)-treated CCI rats than that in the CCI group (*P < 0.05). After AM1241 treatment, the protein expression levels of CB2 receptor was more elevated than that in the CCI group (*P < 0.05).

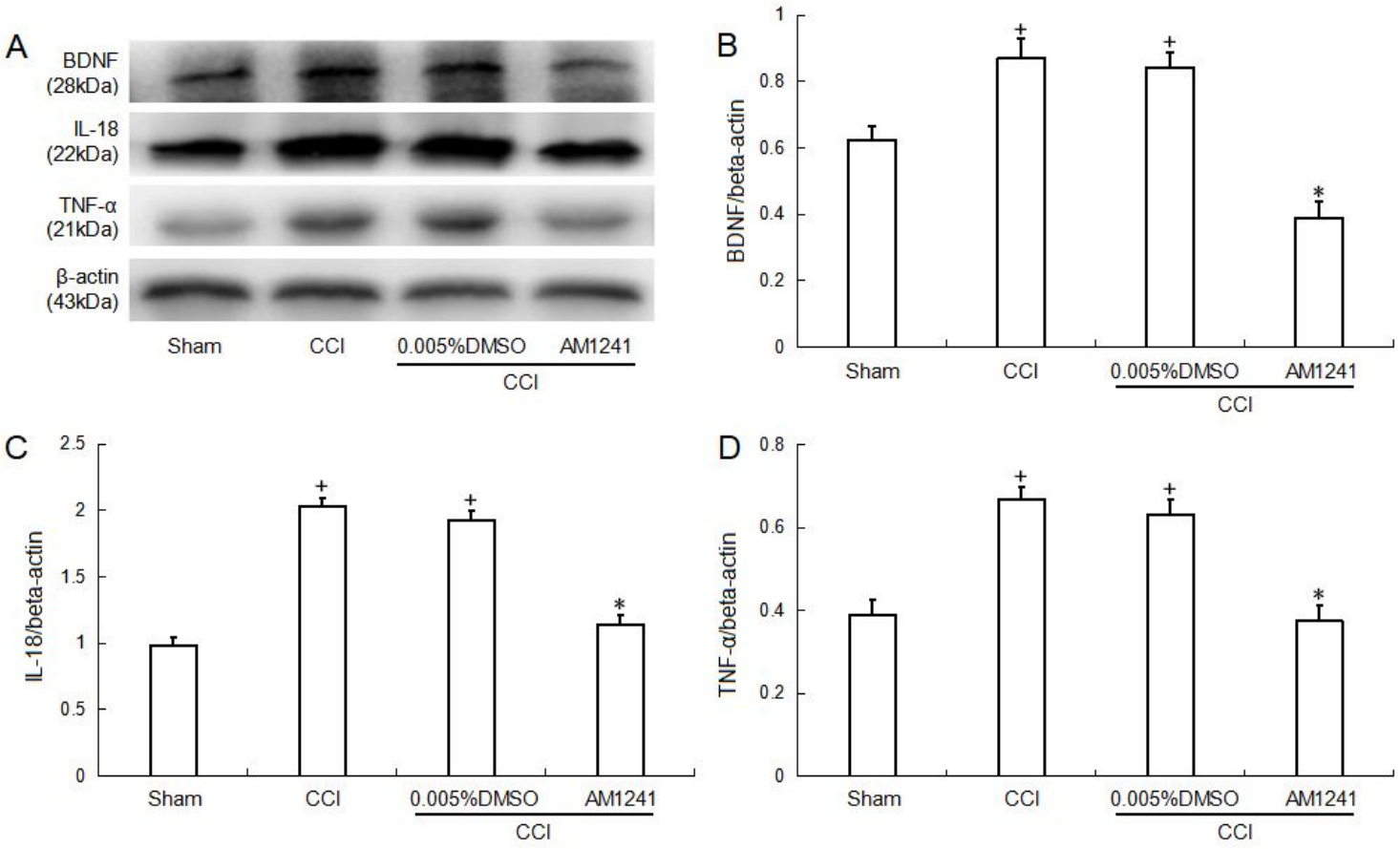

Figure 2

Fig.2 Intrathecal injection of AM1241 (100pM) attenuated CCI-induced increased expression of BDNF, IL-18 and TNF-α in the dorsal spinal cord of CCI rats (n = 5 per group). +P < 0.05, compared with sham groups; *P < 0.05, compared with the CCI or vehicle-treated CCI group; this applies for all three proteins. A: Western blotting image of BDNF, IL-18 and TNF-α expression. B: Western blotting quantitative analysis of the BDNF expression. C: Western blotting quantitative analysis of the IL-18 expression. D: Western blotting quantitative analysis of the TNF-α expression.

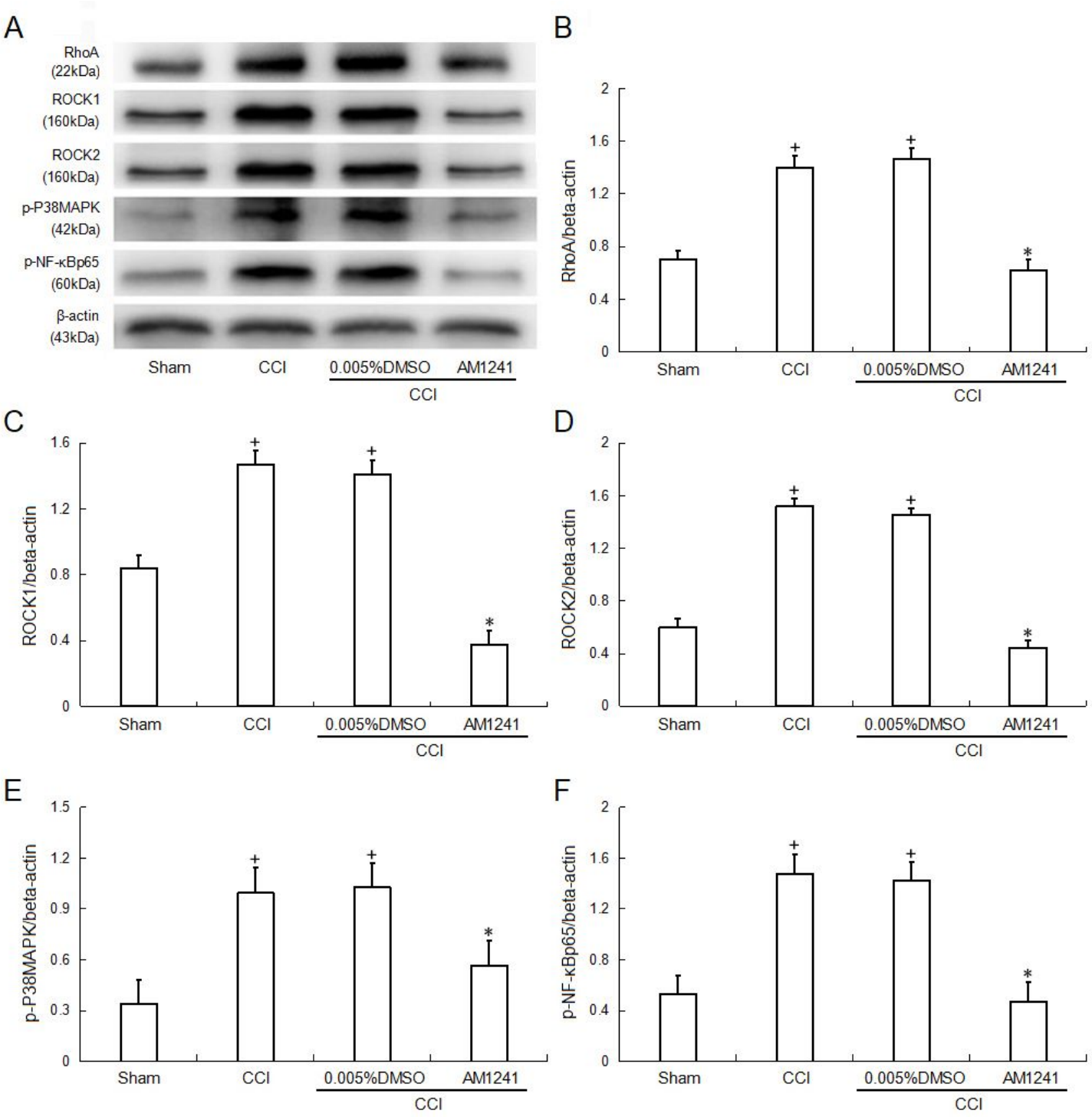

Figure 3

Fig.3 Intrathecal injection of AM1241 (100pM) attenuated CCI-induced increased expression of RhoA, ROCK1/ROCK2 and the activation of p38MAPK and NF-κBp65 in the dorsal spinal cord of CCI rats (n = 5). +P < 0.05, compared with sham groups; *P < 0.05, compared with the CCI or vehicle-treated CCI group; this applies for all five proteins. A：Western blotting image of RhoA, ROCK1, ROCK2, p-p38MAPK and p-NF-κBp65 expression. B: Western blotting quantitative analysis of the RhoA expression. C: Western blotting quantitative analysis of the ROCK1 expression. D: Western blotting quantitative analysis of the ROCK2

expression. E: Western blotting quantitative analysis of the p-p38MAPK expression. F: Western blotting quantitative analysis of the p-NF-κBp65 expression.

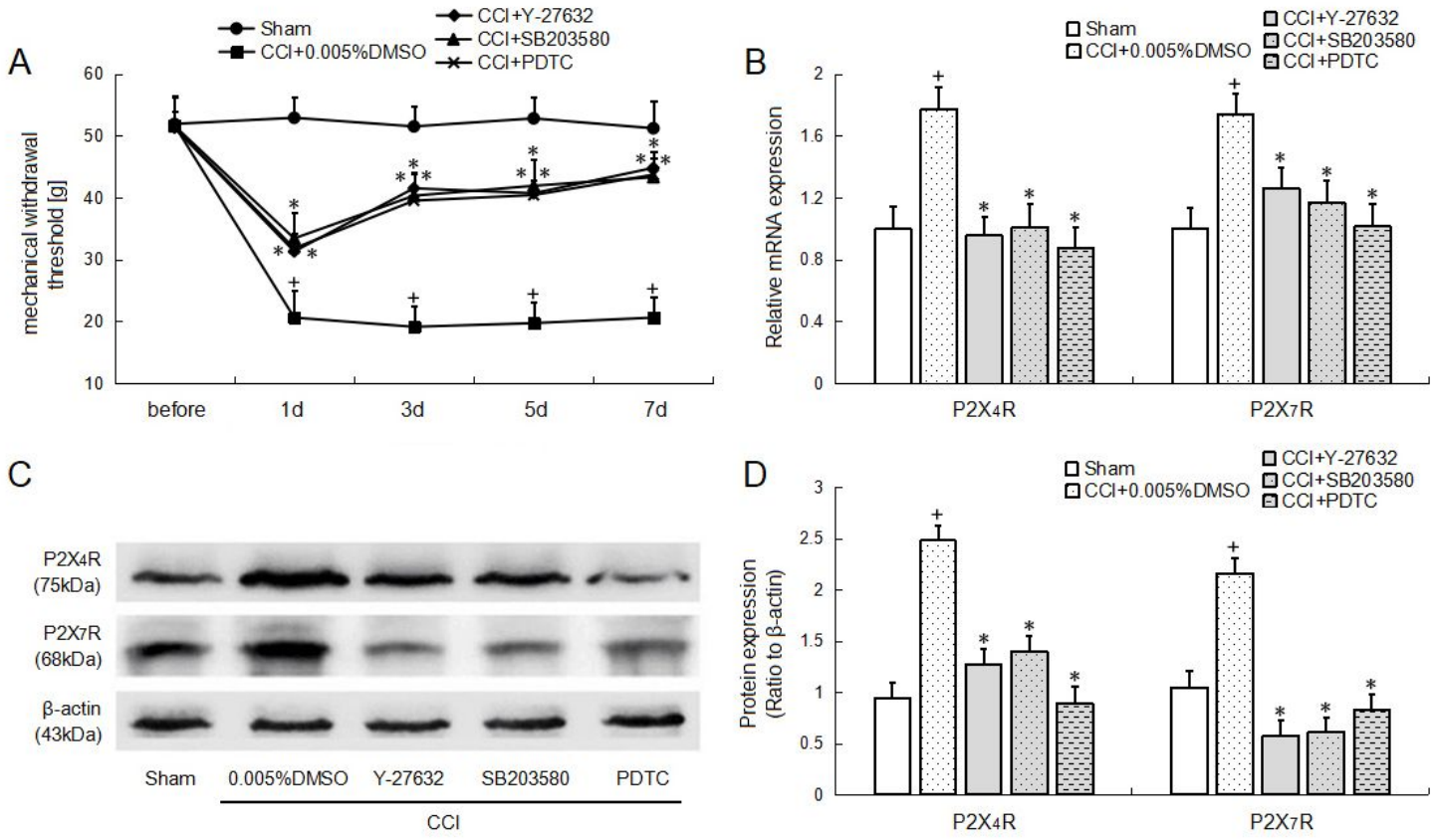

Figure 4

Fig.4 Intrathecal injection of Y-27632 (10-5mol/l), SB203580 (50μM) or PDTC (5μg/10μl) suppressed the CCI-induced mechanical allodynia and the increased expression of P2X4 and P2X7 receptors in dorsal spinal cord. A: Comparison of MWT in the ipsilateral hind paw of the rats (n = 8). CCI reduced MWT from day 1 to 7 after nerve injury (+P <0.05 means comparison with sham group). Pretreated with Y-27632, SB203580 or PDTC significantly inhibited the reduced MWT in CCI rats, respectively (*P<0.05 means comparison with vehicle-treated CCI group). B: RT-PCR results show the expression of P2X4 and P2X7 receptor mRNA expression (n=8 per group). CCI induced the increased expression of P2X4 and P2X7 receptor mRNA, which was obviously suppressed by Y-27632, SB203580 or PDTC (+P <0.05 means comparison with sham group; *P<0.05 means comparison with vehicle-treated CCI group). C: Western blotting image of P2X4 and P2X7R expression. D: Western blotting quantitative analysis of the protein expression of P2X4 and P2X7 receptors in dorsal spinal cord. CCI-induced the increased protein expression of P2X4 and P2X7 receptors were obviously suppressed by Y-27632, SB203580 or PDTC (+P <0.05 means comparison with sham group; *P<0.05 means comparison with vehicle-treated CCI group, n=5).

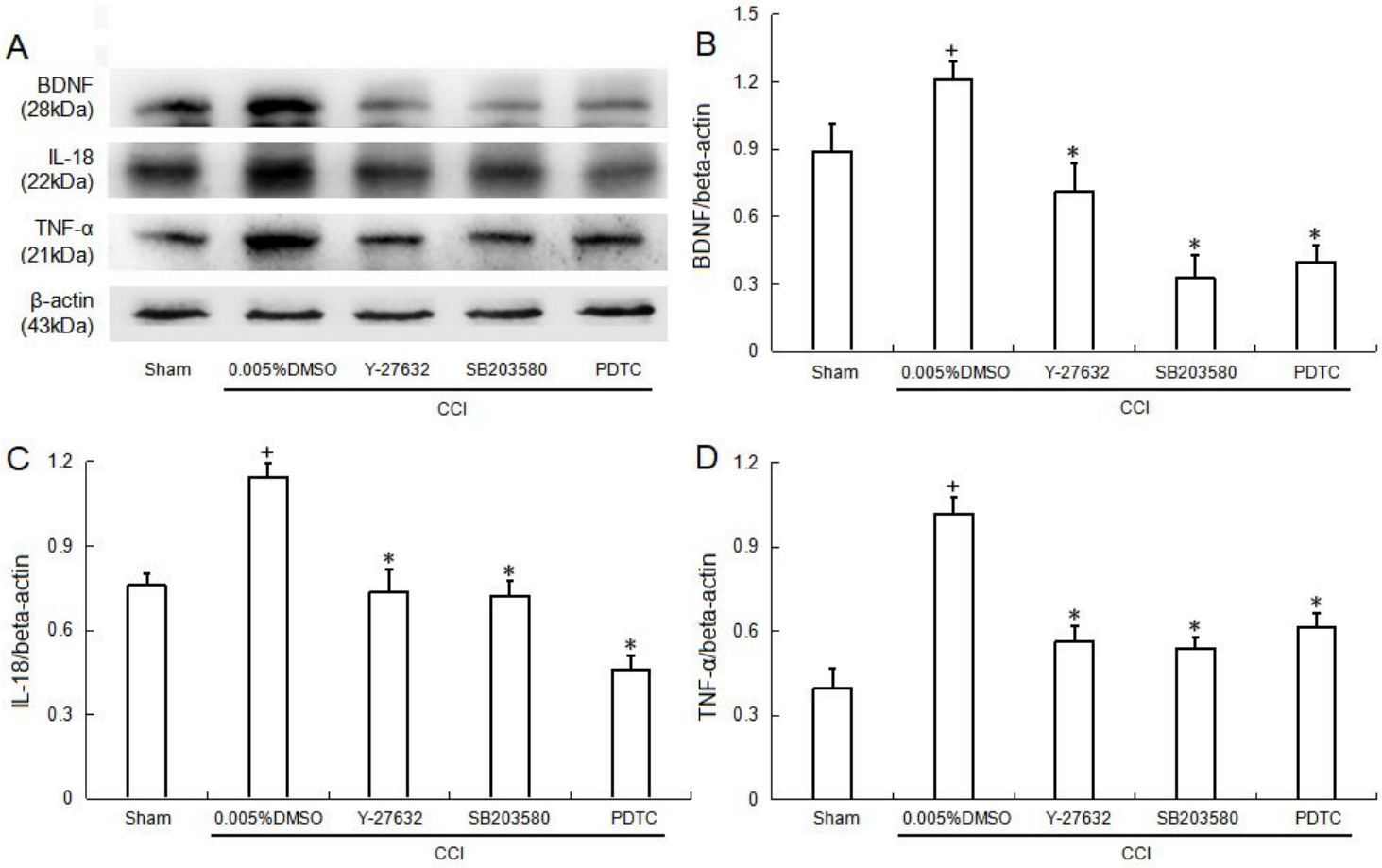

Figure 5

Fig.5 Intrathecal injection of Y-27632 (10-5mol/l), SB203580 (50μM) or PDTC (5μg/10μl) suppressed the CCI-induced the increased expression of BDNF, IL-18 and TNF-α in dorsal spinal cord of CCI rats. +P<0.05 means comparison with sham group; *P<0.05 means comparison with vehicle-treated CCI group; this applies for all three proteins. A: Western blotting image of BDNF, IL-18 and TNF-α expression. B: Western blotting quantitative analysis of the BDNF expression in rat dorsal spinal cord (n=5). C: Western blotting quantitative analysis of the IL-18 expression in rat dorsal spinal cord (n=5). D: Western blotting quantitative analysis of the TNF-α expression in rat dorsal spinal cord (n=5).

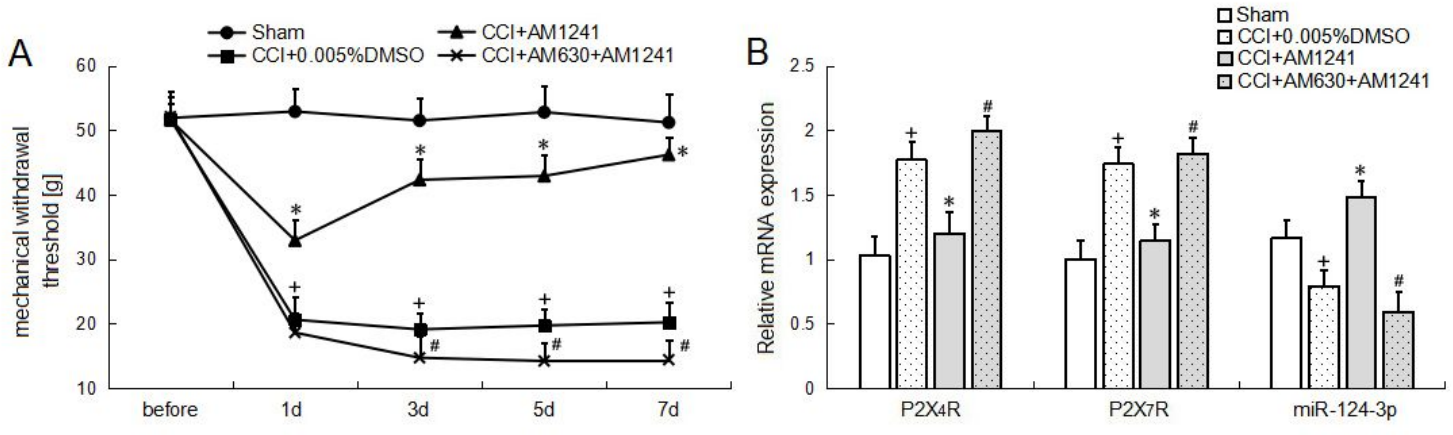

Figure 6

Fig.6 Intrathecal injection of AM1241 (100pM)/AM630 (100μM) changed the CCI-induced mechanical allodynia and the expression of miRNA-124-3p, P2X4 and P2X7 receptors mRNA in dorsal spinal cord of CCI rats. A: Comparison of MWT in the ipsilateral hind paw of rats (n = 6). +P < 0.05, compared with sham group; *P<0.05 means comparison with vehicle-treated CCI group; #P<0.05 means comparison with AM1241-treated CCI group. B: RT-PCR results show the expression of miRNA-124-3p, P2X4 and P2X7 receptors mRNA (n = 6). +P < 0.05, compared with sham group; *P<0.05 means comparison with vehicle-treated CCI group; #P<0.05 means comparison with AM1241-treated CCI group.

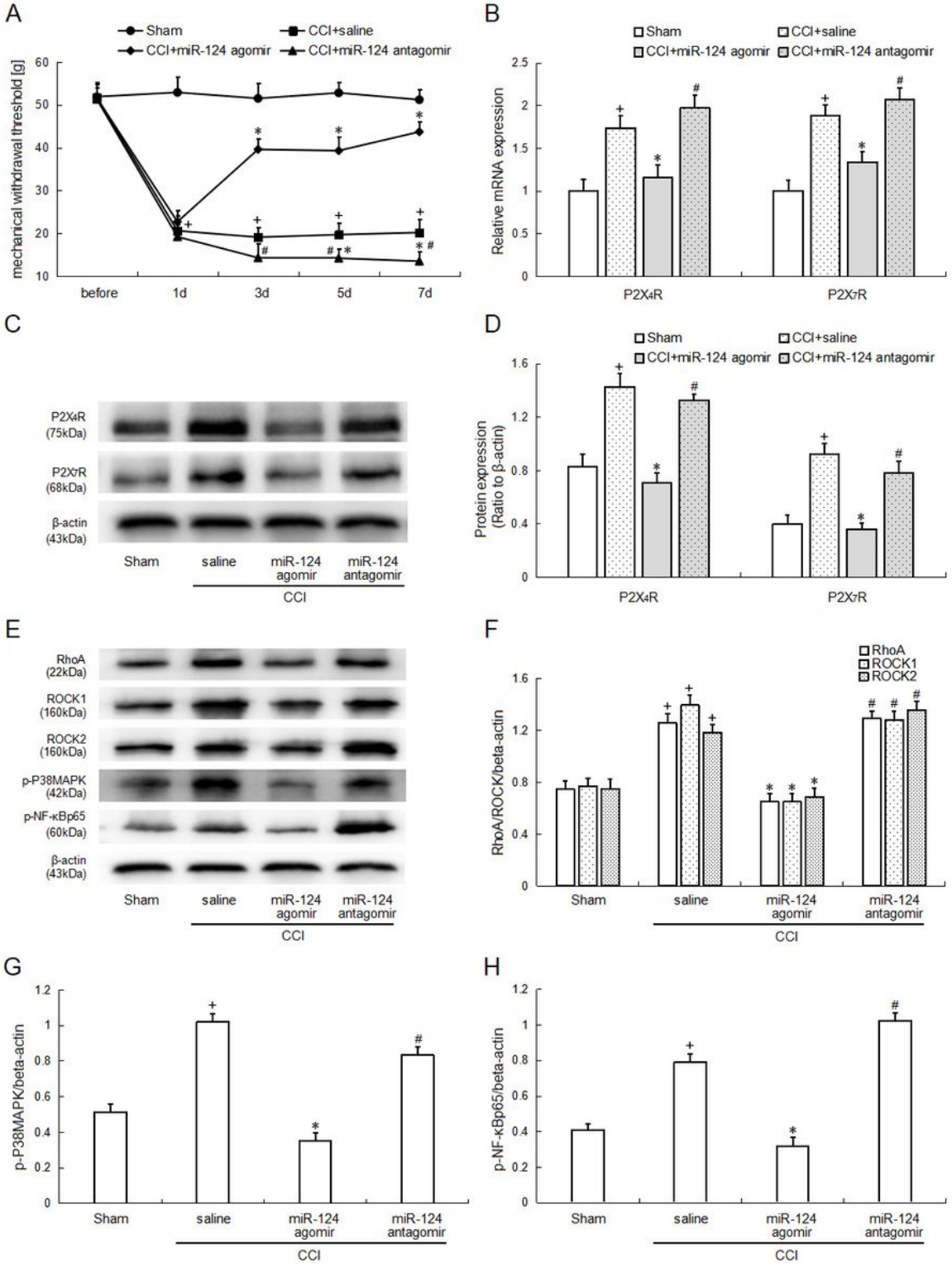

Figure 7

Fig.7 Intrathecal injection of miR-124 agomir (100nM)/antagomir (100nM) changed the CCI-induced mechanical allodynia and the expression of P2X4 and P2X7 receptors, RhoA, ROCK1, ROCK2, p-p38MAPK and p-NF-κBp65 in dorsal spinal cord. A: Comparison of MWT in different groups (n = 6). The MWT in vehicle-treated CCI rats was significantly lower than in the sham group (+P<0.05). MiR-124 agomir (100nM) alleviates pain response after the nerve injury (*P<0.05 means comparison with vehicle-treated CCI group). Intrathecal administration of miR-124-3p antagomir at 100nM enhanced CCI-induced mechanical allodynia at 5 and 7d. B: RT-PCR results show the expression of miRNA-124, P2X4 and P2X7 receptors mRNA (n = 6). +P < 0.05, compared with sham group; *P<0.05 means comparison with vehicle-treated CCI group. #P<0.05 means comparison with miR-124 agomir -treated CCI group. C: Western blotting image of P2X4 and P2X7 receptors protein expression. D: Western blotting quantitative analysis of the P2X4 and P2X7 receptor protein expression in rat dorsal spinal cord. +P<0.05 means comparison with sham group; *P<0.05 means comparison with vehicle-treated CCI group; #P<0.05 means comparison with miR-124 agomir -treated CCI group. (n=5). E: Western blotting image of RhoA, ROCK1 and ROCK2, p-p38MAPK and p-NF-kappaBp65 expression. F: Western blotting quantitative analysis of the RhoA, ROCK1 and ROCK2 expression in rat dorsal spinal cord. +P<0.05 means comparison with sham group; *P<0.05 means comparison with vehicle-treated CCI group; #P<0.05 means comparison with miR-124 agomir -treated CCI group. (n=5). G: Western blotting quantitative analysis of the p-p38MAPK expression in rat dorsal spinal cord. +P<0.05 means comparison with sham group; *P<0.05 means comparison with vehicle-treated CCI group; #P<0.05 means comparison with miR-124 agomir -treated CCI group. (n=5). H: Western blotting quantitative analysis of the p-NF-kappaBp65 expression in rat dorsal spinal cord. +P<0.05 means comparison with sham group; *P<0.05 means comparison with vehicle-treated CCI group; #P<0.05 means comparison with miR-124 agomir -treated CCI group. (n=5).

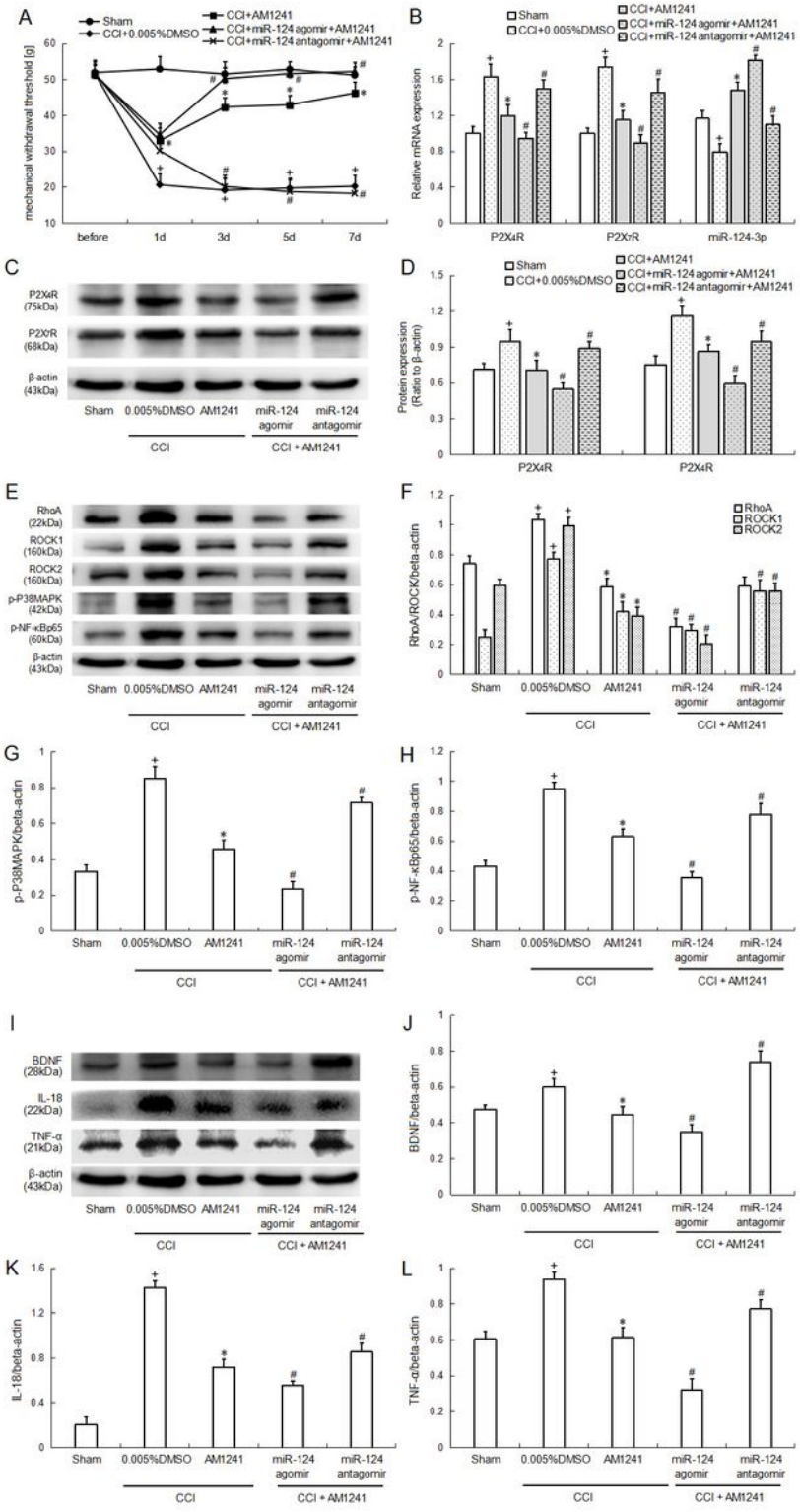

Figure 8

Fig.8 Intrathecal injection of miRNA-124-3p agomir/antagomir changed the CCI-induced mechanical allodynia and some protein expression in AM1241-treated CCI rats. A: Comparison of MWT in every groups (n = 6). +P < 0.05, compared with sham group; *P<0.05 means comparison with vehicle-treated CCI group. #P<0.05 means comparison with AM1241-treated CCI group. B: RT-PCR results show the expression of miRNA-124-3p, P2X4 and P2X7 receptors mRNA (n = 6). +P < 0.05, compared with sham

group; *P<0.05 means comparison with vehicle-treated CCI group; #P<0.05 means comparison with AM1241-treated CCI group. C: Western blotting image of P2X4 and P2X7R expression. D: Western blotting quantitative analysis of the P2X4 and P2X7R expression in dorsal spinal cord. +P<0.05, compared with sham group; *P<0.05, compared with vehicle-treated CCI group; #P<0.05 means comparison with AM1241-treated CCI group (n=5). E: Western blotting image of RhoA, ROCK1 and ROCK2, p-P38MAPK and p-NF-κBp65 expression. F: Western blotting quantitative analysis of the RhoA, ROCK1 and ROCK2 expression in dorsal spinal cord. +P<0.05, compared with sham group; *P<0.05, compared with vehicle-treated CCI group; #P<0.05 means comparison with AM1241-treated CCI group (n=5). G: Western blotting quantitative analysis of the p-p38MAPK expression in dorsal spinal cord. +P<0.05 means comparison with sham group; *P<0.05 means comparison with vehicle-treated CCI group; #P<0.05 means comparison with AM1241-treated CCI group (n=5). H: Western blotting quantitative analysis of the p-NF-κBp65 expression in rat dorsal spinal cord. +P<0.05 means comparison with sham group; *P<0.05 means comparison with vehicle-treated CCI group; #P<0.05, compared with AM1241-treated CCI group (n=5). I: Western blotting image of the BDNF, IL-18 and TNF-α expression in rat dorsal spinal cord. J: Western blotting quantitative analysis of the BDNF expression in dorsal spinal cord. +P<0.05, compared with sham group; *P<0.05, compared with vehicle-treated CCI group; #P<0.05 means comparison with AM1241-treated CCI group (n=5). K: Western blotting quantitative analysis of the IL-18 expression in dorsal spinal cord. +P<0.05, compared with sham group; *P<0.05, compared with vehicle-treated CCI group; #P<0.05 means comparison with AM1241-treated CCI group (n=5). L: Western blotting quantitative analysis of the TNF-α expression in dorsal spinal cord. +P<0.05, compared with sham group; *P<0.05, compared with vehicle-treated CCI group; #P<0.05 means comparison with AM1241-treated CCI group (n=5).